Gene Identification for the cblD Defect of Vitamin B12Metabolism

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Abstract: Background Vitamin B12 (cobalamin) is an essential cofactor in several metabolic pathways. Intracellular conversion of cobalamin to its two coenzymes, adenosylcobalamin in mitochondria and methylcobalamin in the cytoplasm, is necessary for the homeostasis of methylmalonic acid and homocysteine. Nine defects of intracellular cobalamin metabolism have been defined by means of somatic complementation analysis. One of these defects, the cblD defect, can cause isolated methylmalonic aciduria, isolated homocystinuria, or both. Affected persons present with multisystem clinical abnormalities, including developmental, hematologic, neurologic, and metabolic findings. The gene responsible for the cblD defect has not been identified. Methods We studied seven patients with the cblD defect, and skin fibroblasts from each were investigated in cell culture. Microcell-mediated chromosome transfer and refined genetic mapping were used to localize the responsible gene. This gene was transfected into cblD fibroblasts to test for the rescue of adenosylcobalamin and methylcobalamin synthesis. Results The cblD gene was localized to human chromosome 2q23.2, and a candidate gene, designated MMADHC (methylmalonic aciduria, cblD type, and homocystinuria), was identified in this region. Transfection of wild-type MMADHC rescued the cellular phenotype, and the functional importance of mutant alleles was shown by means of transfection with mutant constructs. The predicted MMADHC protein has sequence homology with a bacterial ATP-binding cassette transporter and contains a putative cobalamin binding motif and a putative mitochondrial targeting sequence. Conclusions Mutations in a gene we designated MMADHC are responsible for the cblD defect in vitamin B12 metabolism. Various mutations are associated with each of the three biochemical phenotypes of the disorder.

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ORIGINAL ARTICLE

Gene Identification for the cblD Defect of Vitamin B_{12} Metabolism

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ABSTRACT

BACKGROUND

Vitamin B_{12} (cobalamin) is an essential cofactor in several metabolic pathways. Intracellular conversion of cobalamin to its two coenzymes, adenosylcobalamin in mitochondria and methylcobalamin in the cytoplasm, is necessary for the homeostasis of methylmalonic acid and homocysteine. Nine defects of intracellular cobalamin metabolism have been defined by means of somatic complementation analysis. One of these defects, the cblD defect, can cause isolated methylmalonic aciduria, isolated homocystinuria, or both. Affected persons present with multisystem clinical abnormalities, including developmental, hematologic, neurologic, and metabolic findings. The gene responsible for the cblD defect has not been identified.

METHODS

We studied seven patients with the cblD defect, and skin fibroblasts from each were investigated in cell culture. Microcell-mediated chromosome transfer and refined genetic mapping were used to localize the responsible gene. This gene was transfected into cblD fibroblasts to test for the rescue of adenosylcobalamin and methylcobalamin synthesis.

RESULTS

The cblD gene was localized to human chromosome 2q23.2, and a candidate gene, designated *MMADHC* (methylmalonic aciduria, cblD type, and homocystinuria), was identified in this region. Transfection of wild-type *MMADHC* rescued the cellular phenotype, and the functional importance of mutant alleles was shown by means of transfection with mutant constructs. The predicted MMADHC protein has sequence homology with a bacterial ATP-binding cassette transporter and contains a putative cobalamin binding motif and a putative mitochondrial targeting sequence.

CONCLUSIONS

Mutations in a gene we designated *MMADHC* are responsible for the cblD defect in vitamin B_{12} metabolism. Various mutations are associated with each of the three biochemical phenotypes of the disorder.

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Vitamin B_{12} (cobalamin) is essential for normal development and survival in humans and must be obtained from animal products or supplements. Inside the cell, it is converted to two active cofactors, adenosylcobalamin and methylcobalamin (Fig. 1).[1] Adenosylcobalamin is the coenzyme for mitochondrial methylmalonyl–coenzyme A mutase, which converts L-methylmalonyl–coenzyme A to succinyl–coenzyme A and is involved in catabolism of odd-chain fatty acids and some amino acids. Methylcobalamin is the coenzyme for cytosolic methionine synthase, which converts homocysteine to methionine and is essential for normal one-carbon metabolism, which is in turn involved in vital cellular processes such as methylation and DNA synthesis.[1] Disturbances of cobalamin-cofactor synthesis due to acquired or inherited alterations result in elevated levels of homocysteine and methylmalonic acid, which are associated with multisystem clinical abnormalities similar to those seen in patients with severe nutritional vitamin B_{12} deficiency, including lethargy, hypotonia, developmental delay, seizures, and megaloblastic anemia.

Inborn errors of cobalamin-cofactor synthesis represent a heterogeneous and important group of rare disorders. Intracellular cobalamin metabolism involves multiple steps between the lysosomal release of cobalamin and the synthesis of adenosylcobalamin in the mitochondria and methylcobalamin in the cytosol. To date, nine distinct defects of this pathway have been defined in vitro with the use of somatic complementation analysis. The complementation groups thus identified have been designated cblA, cblB, cblC, cblD, cblE, cblF, cblG, cblH, and mut (Fig. 1).[2,3] The responsible genes are known except for cblD, cblF, and cblH, although the function of some of the associated proteins is not clear. The cblC and cblF disorders cause combined homocystinuria and methylmalonic aciduria; cblA, cblB, cblH, and mut cause isolated methylmalonic aciduria; and cblE and cblG cause isolated homocystinuria. The cblD defect (Online Mendelian Inheritance in Man number, 277410)[2] is puzzling in that some patients have combined methylmalonic aciduria and homocystinuria (called "cblD original" by Suormala et al.[2] but herein called "cblD-combined"), some have only isolated homocystinuria (called "cblD-variant 1"[2] or, herein, "cblD-homocystinuria"), and others have only methylmalonic aciduria ("cblD-variant 2"[2] or, herein, "cblD–methylmalonic aciduria"). Here, we describe our identification of the cblD gene, confirmation of its identity by using biochemical and molecular studies, and demonstration of functionally significant mutations in patients with the cblD defect.

METHODS

PATIENTS AND CELL LINES

The study was performed between 2004 and 2007. Seven unrelated patients with the cblD defect were studied. Written informed consent was obtained from the parents of each patient, and patients who could give assent did so. The study was approved by the local ethics committee.

Cultures of skin fibroblasts were obtained for diagnostic purposes, and referring physicians approved the use of the samples for our investigation of the origin of the disease. We immortalized fibroblasts using the plasmid pRNS1[4] and electroporation (Gene Pulser II, BioRad). Immortalization did not markedly affect cellular function (data not shown).

The functional integrity of the methylmalonic-acid pathway was evaluated by measuring the degree of incorporation of [^{14}C]propionate into macromolecules and formation of adenosylcobalamin from [^{57}Co]cyanocobalamin, as described previously.[2] The integrity of methionine synthase in the homocysteine pathway was defined as the formation of methionine from [^{14}C]formate and synthesis of methylcobalamin from [^{57}Co]cyanocobalamin.[2]

Somatic complementation analysis[2] confirmed that all fibroblast cell lines from patients belonged to the cblD group (including one that had been used to define cblD[5]). A fibroblast cell line from one of the seven patients had previously been assigned to a new complement group (cblH) by Watkins et al.,[6] but we show here that it in fact belongs to the cblD complement group (Fig. 1 in the Supplementary Appendix, available with the full text of this article at www.nejm.org).

MICROCELL-MEDIATED CHROMOSOME TRANSFER

Mouse–human monochromosomal hybrid cell lines (donor cells), each carrying a single human chromosome tagged with a hygromycin resistance gene,[7] were used to serially transfect one of the immortalized cblD-homocystinuria cell lines (recipient cells) with the use of microcell-mediated chromosome transfer, as described previously[8]

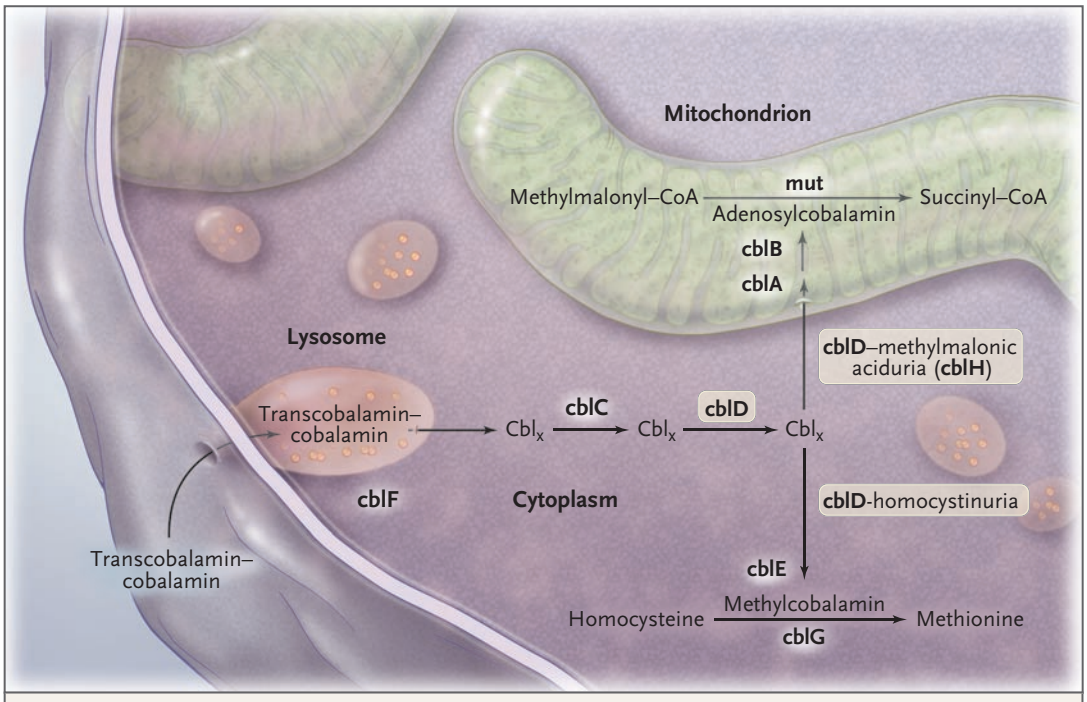


Figure 1. Intracellular Cobalamin Metabolism and Its Defects.

To date, nine complementation-group defects of the cobalamin pathway have been described. Cobalamin bound to transcobalamin enters the cell by means of lysosome-mediated endocytosis and is released through proteolysis. Export from the lysosome into the cytoplasm is defective in patients with the cblF defect. The steps in the cytosol after lysosomal release are still unclear but are defined by the complementation groups cblC and cblD. In addition, the exact form of cobalamin at this stage is unclear (as indicated by "Cbl$_x$"). In the cytoplasm, cobalamin is reductively methylated by methionine synthase reductase (cblE) to methylcobalamin, the cofactor for methionine synthase (cblG). After its transport into the mitochondrion, cobalamin is converted to adenosylcobalamin, the cofactor for methylmalonyl–coenzyme A (CoA) mutase (mut), by cobalamin adenosyltransferase (cblB). The exact role of the protein associated with the cblA complementation group is unclear. Our studies show that the cblD protein constitutes a branch point between the cytosolic and mitochondrial pathways, controlled by the cblD–methylmalonic aciduria variant and the cblD-homocystinuria variant, respectively. We also show that the cblH complementation group is identical to the cblD–methylmalonic aciduria group.

(also see Table 1 in the Supplementary Appendix). Cells containing each transfected human chromosome were selected on the basis of growth in medium containing hygromycin. Colonies were subcultured and assayed for methylcobalamin synthesis. Those that showed such synthesis, and thus the rescue of cellular function, were defined as positive colonies; those that did not were defined as negative colonies.

GENE MAPPING

Positive colonies were tested for contamination by mouse DNA with the use of the mouse-specific microsatellite markers BAM5 and RSINE1 SINE/B4.[9,10] Positive colonies that were free of mouse DNA contamination and negative colonies then were used in the fine mapping of the donor chromosome, by means of microsatellite markers, to define the chromosomal segment containing the putative gene corresponding to the cblD defect. The presence of these microsatellite markers was tested with the use of the polymerase-chain-reaction (PCR) assay, involving fluorescently labeled primers. Products were visualized in an automated sequencer (ABI Prism 3100, Applied Biosystems) and analyzed with Genotyper software (version 2.5, Applied Biosystems). The chromosomal region defined by the microsatellite markers was examined for candidate genes, and a suitable candidate was selected on the basis of sequence characteristics.

DNA SEQUENCING AND MUTATION ANALYSIS

Total RNA was extracted from cultured fibroblasts with the use of the RNeasy Kit (Qiagen), and

full-length complementary DNA (cDNA) for the candidate gene was amplified by means of a reverse-transcriptase PCR (RT-PCR) assay involving specific primers and was sequenced according to the ABI BigDye method (Applied Biosystems). To confirm mutations identified in the RT-PCR products, the corresponding exons were amplified through the PCR assay from genomic DNA, with the use of flanking intronic primers, and were sequenced.

EXPRESSION OF CANDIDATE GENE cDNA IN FIBROBLASTS

Constructs containing wild-type and mutant cDNA sequences for the candidate gene were prepared in pTracer-CMV2 or pcDNA3.2/V5 expression vectors (Invitrogen), as described previously.[11] Constructs were transfected into immortalized fibroblasts by means of electroporation. Transfection efficiency was 5 to 22%, as determined by estimating the proportion of cells coexpressing the green fluorescent protein from the pTracer construct. Rescue of cellular function was tested by measuring methionine and methylcobalamin synthesis or adenosylcobalamin synthesis.

STATISTICAL ANALYSIS

We tested the statistical significance of the data on rescue of function by using the unpaired t-test (two-tailed), with Welch's correction for unequal variances, and GraphPad Prism software (version 4). P values less than 0.05 were considered to indicate statistical significance.

RESULTS

CLINICAL DATA

The clinical features of the seven patients are shown in Table 1. Two patients had the isolated homocystinuria phenotype, and two had the isolated methylmalonic aciduria phenotype. These four patients have been described previously (Table 1). The three other patients, two of whom are newly described in this study, had the combined phenotype (methylmalonic aciduria and homocystinuria).

GENE LOCALIZATION

To identify the chromosome containing the cblD gene, we transfected immortalized cblD-homocystinuria cells (from Patient 2) with individual human chromosomes contained in mouse–human single-chromosome hybrid cell lines using microcell-mediated chromosome transfer. We then tested the resulting cell lines for rescue of function by measuring methylcobalamin synthesis. Transfection with chromosome 2 yielded 48 colonies that showed correction of methylcobalamin synthesis (positive colonies) and 24 colonies that did not (negative colonies) (Table 1 in the Supplementary Appendix). The positive colonies were screened for mouse-specific sequences, and four were found to be free of contamination with mouse DNA.

A panel of 38 microsatellite markers spanning chromosome 2 was used to perform fine mapping of the chromosome fragments from the 4 positive colonies and 22 of the negative colonies (see Table 2 in the Supplementary Appendix). This fine-mapping procedure identified a 10.2-Mb DNA segment between markers D2S150 and D2S2324 that was present in DNA from positive colonies but absent in all the negative colonies (Fig. 2 in the Supplementary Appendix). This mapped the cblD gene to the chromosome region 2q22.1–2q23.3 (Fig. 2A), which contains 28 genes (according to the deCODE genetic map; for details, see the accession numbers and URLs in Note 1 in the Supplementary Appendix). The *C2orf25* gene was selected as a candidate because of its homology[14] to a putative mitochondrial ATPase component of a bacterial ATP-binding cassette (ABC) transporter (YP_218380) and because cobalamin transport in bacteria is facilitated by an ABC transport system.[15]

MUTATION ANALYSIS

The full-length cDNA for the candidate gene *C2orf25* was amplified by means of RT-PCR and specific primers (Table 3 in the Supplementary Appendix). The *C2orf25* gene was then sequenced from cDNA from each of the seven patients with the cblD defect, and mutations for each patient were identified. These mutations were further confirmed with the use of PCR amplification of the appropriate exons from genomic DNA, involving flanking intronic primers (Table 3 in the Supplementary Appendix). We identified nine mutations, with two mutant alleles in each patient (Table 1 and Fig. 2B).

Five of the mutations we identified are predicted to lead to a premature stop codon, resulting in a truncated protein: two nonsense mutations (160C→T, 748C→T), two deletions (57_64del, 696+1_4del), and one duplication (419dupA). The 696+1_4del splice-site mutation was shown by

Table 1. Mutations in Complementary DNA in Seven Patients with the cblD Defect.*

Patient No. and Mutant Alleles†	Predicted Amino Acid Mutations	Ethnic Group‡	Sex	Parental Consanguinity	Biochemical Phenotype	Age at Diagnosis	Clinical Findings Leading to Diagnosis	Study
1								
776T→C (both alleles)	Leu259→Pro	Irish	M	Yes	Homocystinuria	6 Yr	Global developmental delay, spastic ataxia, gait problems, delayed visual evoked potentials, increased mean corpuscular volume	Suormala et al.[2] (Case 1)
2								
545C→A, 746A→G	Thr182→Asn, Tyr249→Cys	Italian	M	No	Homocystinuria	3 Mo	Hypotonia, nystagmus, dystonia, seizures, megaloblastic anemia	Suormala et al.[2] (Case 2)
3								
57_64delCTCTTTAG (both alleles)	Cys19fsX20	Indian	M	No	Methylmalonic aciduria	Unknown§	Preterm birth at 32 wk of gestation, grade II respiratory distress syndrome, necrotizing enterocolitis, neonatal convulsions	Suormala et al.[2] (Case 3)
4								
160C→T, 307_324dup	Arg54X, Leu103_Ser108dup	Haitian	M	No	Methylmalonic aciduria	11 Mo	Severe ketotic coma, dehydration, hyperammonemia, leukopenia, thrombocytopenia	Cooper et al.[3]
5								
748C→T (both alleles)	Arg250X	Spanish-American	M	Yes	Methylmalonic aciduria and homocystinuria	14 Yr	Acute psychotic episode, marfanoid appearance, nystagmus, increased mean corpuscular volume, mild mental retardation	Goodman et al.[12] (Case 1) and Carmel and Goodman[13] (Patient J.R.)
6								
419dupA (both alleles)	Tyr140X	Scandinavian	F	Unknown¶	Methylmalonic aciduria and homocystinuria	3 Mo	Developmental delay, seizures, megaloblastic anemia	This study
7								
696+1_4delGTGA (both alleles)	Phe204_Ala232del	Italian	M	Yes	Methylmalonic aciduria and homocystinuria	22 Days	Poor feeding, encephalopathy, seizures, increased mean corpuscular volume	This study

* Fibroblast cultures from Patients 4 and 5 were obtained from the Repository for Mutant Human Cell Strains, Montreal Children's Hospital (where the patients were identified as WG1437 [cblH] and WG1220 [the original patient with the cblD defect], respectively). Fibroblast cultures from Patients 6 and 7 were supplied by Dr. B. Woldseth (Oslo, Norway) and Dr. D. Ketteridge (Adelaide, Australia), respectively.

† Mutation numbering is based on the complementary DNA sequence (NM_015702) with +1 corresponding to the A of the ATG translation-initiation codon. The 696+1_4delGTGA mutation results in exon 7 being skipped.

‡ Ethnic group was reported by the parents of the patients.

§ Elevated excretion of methylmalonic acid was detected and treated shortly after birth, but enzymatic diagnosis was performed only at the age of 8 months.

¶ The parents of this patient were from the same village.

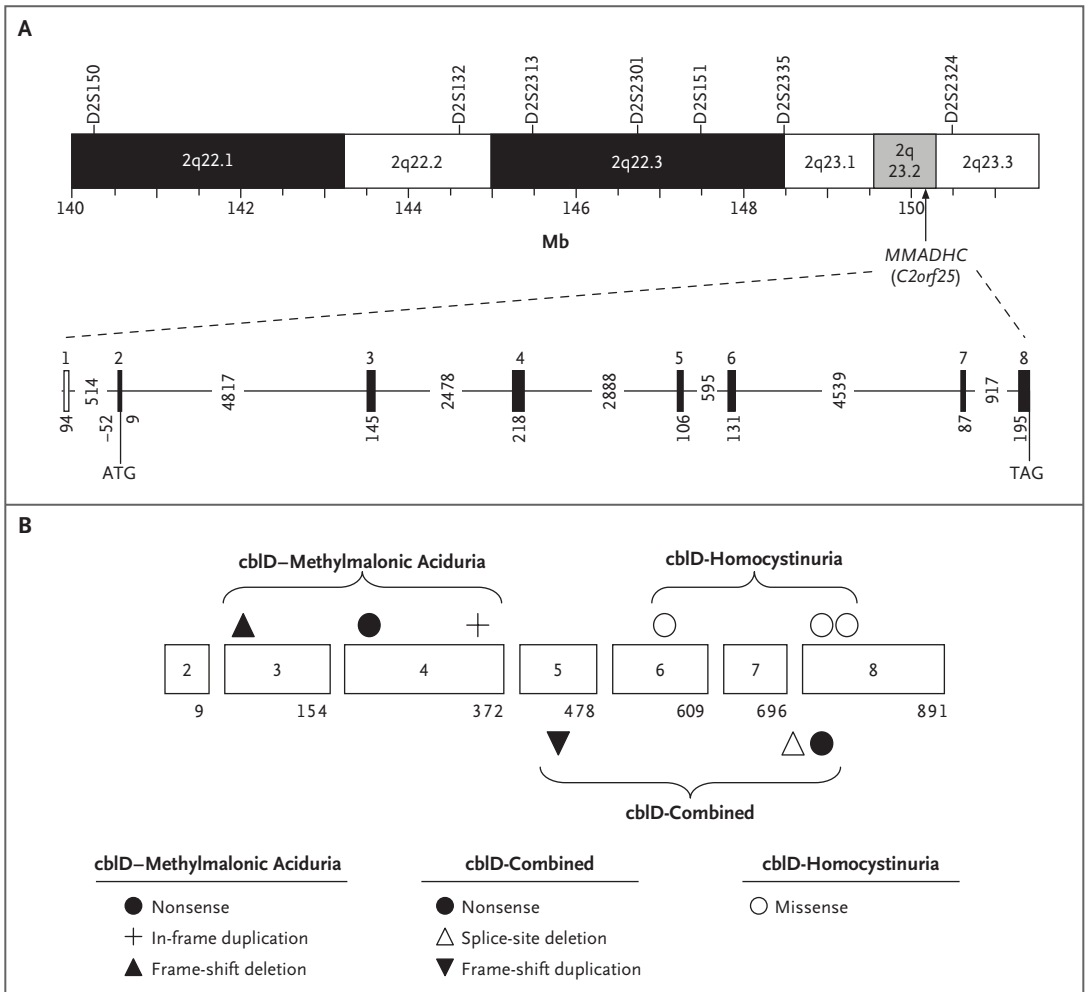


Figure 2. Location, Structural Organization, and Mutations of *MMADHC* (Methylmalonic Aciduria, cblD Type, and Homocystinuria).

Panel A shows the fragment of chromosome 2, between markers D2S150 and D2S2324, that was identified by means of microcell-mediated chromosome transfer and microsatellite mapping. This fragment is 10.2 Mb in length. The position of the markers is indicated according to the National Center for Biotechnology Information's human reference sequence, build 36.1. *MMADHC* (currently named *C2orf25*; locus NM_015702) is located in chromosome 2 (genomic sequence NT_005403) and maps to chromosome region 2q23.2. The gene consists of eight exons. Three in-frame stop codons precede the first initiation codon (ATG) in exon 2. Exon 1 and the 5′ end of exon 2 are not translated. Exons 2 through 8 encode an 891-bp message, from the initiation codon to the TAG termination codon, predicted to encode a polypeptide of 296 amino acids with a predicted molecular mass of 32.8 kD. The coding regions are indicated as black vertical bars with exon numbers above and the size in base pairs below; intron sizes, in base pairs, are indicated between the exons. Panel B represents composite complementary DNA (cDNA) for *MMADHC* and shows the location of mutations, grouped according to the cblD phenotype. Exons are represented by the numbered boxes, and the numbers below the boxes indicate the position within the cDNA sequence of the last nucleotide of each exon.

means of RT-PCR to cause the skipping of exon 7. This mutation was present in the homozygous state in Patient 7 and was found in the heterozygous state in both parents and in an at-risk fetus in this family (data not shown).

One in-frame duplication (307_324dup) was identified. This mutation is predicted to add six amino acids to the protein product.

Three mutations causing a single amino acid change were found (545C→A, 746A→G, and 776T→C), each occurring in a region of the gene that is highly conserved among species (Fig. 3).

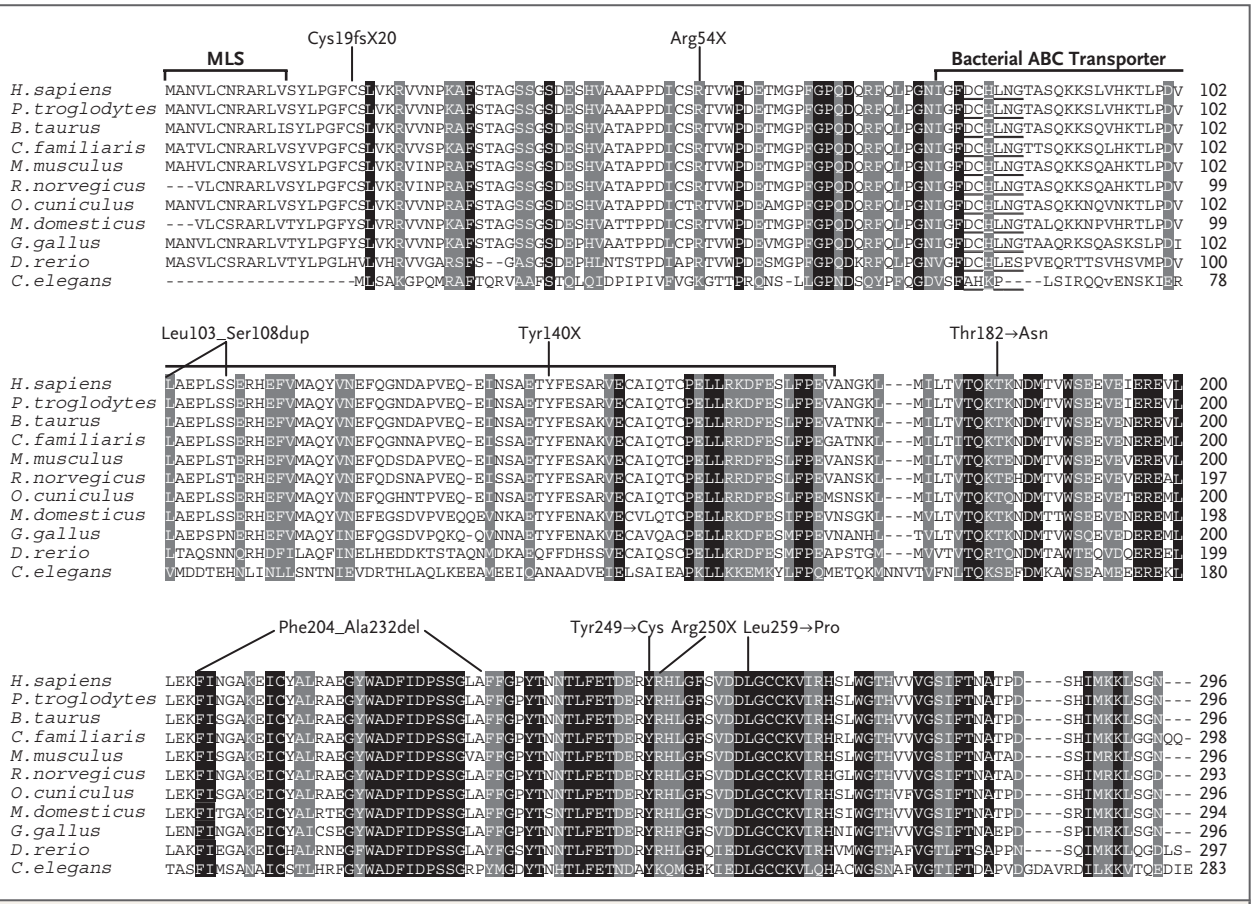


Figure 3. Evolutionary Conservation of MMADHC (Methylmalonic Aciduria, cblD Type, and Homocystinuria).

Amino acid sequence alignment for MMADHC and its orthologues was performed with the use of ClustalW software. Residues that are identical in all species listed are shown in black; residues with conservative substitutions are shown in gray. The portion of the sequence with genetic identity to a bacterial hypothetical mitochondrial ATP-binding cassette (ABC) transporter is indicated. The amino acids constituting the putative cobalamin binding motif are underlined (residues 81–86). Dashes represent amino acids that are absent in the protein of the particular species. The value along the right side of the sequences indicates the number of the last amino acid shown in the row. MLS denotes the putative mitochondrial leader sequence, as predicted by means of the Mitoprot II program. Sequences are shown for the following species (top to bottom in each group): *Homo sapiens, Pan troglodytes, Bos taurus, Canis familiaris, Mus musculus musculus, Rattus norvegicus, Oryctolagus cuniculus, Mus musculus domesticus, Gallus gallus, Danio rerio, and Caenorhabditis elegans.*

These three missense mutations occurred in patients of European origin and did not occur in 100 control chromosomes from subjects with the same ethnic backgrounds, substantially reducing the likelihood that these are common polymorphisms. Parental DNA, used to rule out a deletion in the homozygous patients, was available from only one family.

TISSUE EXPRESSION AND PROTEIN CHARACTERIZATION

We searched databases of known expressed sequences of the human genome to ascertain the tissue expression of the *C2orf25* gene. This gene appears to be expressed at high levels in most tissues (Note 2 in the Supplementary Appendix). To detect possible multiple transcripts, we amplified *C2orf25* from fibroblast messenger RNA (mRNA), by using RT-PCR and various primers. We found only a single cDNA product, with a coding region of 891 bp, encoding a putative polypeptide of 296 amino acids and a predicted molecular weight of 32.8 kD (Fig. 3). Residues 1 through 12 constitute a possible mitochondrial leader sequence, which suggests targeting of the expressed protein to mitochondria.[16] Residues 81 though 86 match the

vitamin B_{12} binding motif, Asp–Xaa–His–Xaa–Xaa–Gly, constituting a putative binding site for cobalamin.[17]

EXPRESSION OF HUMAN *C2orf25* cDNA IN FIBROBLASTS

To prove that the *C2orf25* gene is responsible for the cblD phenotype, we tested the ability of wild-type and mutant constructs to rescue cellular function (Fig. 4). The wild-type construct rescued methionine synthesis and methylcobalamin synthesis, which were both restored to 52 to 100% of the control values in both cblD-homocystinuria and cblD-combined fibroblasts with the use of the pTracer vector (Fig. 4A and 4B). Adenosylcobalamin synthesis was not reproducibly corrected with the use of the pTracer wild-type construct. To test for rescue of adenosylcobalamin synthesis, we repeated the experiment with the DNA3.2/V5 vector containing the wild-type construct and showed restoration of adenosylcobalamin synthesis to 68% of the control value in a cblD-combined cell line (Fig. 4C).

Constructs containing the missense alleles associated with isolated homocystinuria (545C→A, 746A→G, and 776T→C) did not restore methionine or methylcobalamin synthesis in either cblD-homocystinuria or cblD-combined cells, confirming that these mutant alleles cause the homocystinuria phenotype (Fig. 4A and 4B). Both constructs containing the mutations found in isolated methylmalonic aciduria (57_64del and 160C→T) rescued the synthesis of methylcobalamin (Fig. 4B).

Together, these findings suggest that mutations in the *C2orf25* gene are responsible for the cblD defect. We have therefore designated the gene *MMADHC*, or methylmalonic aciduria, cblD type, and homocystinuria (to indicate either the isolated phenotype or the combined phenotype).

DISCUSSION

We show evidence that mutations of the gene *C2orf25*, which we have designated *MMADHC*, cause the cblD defect in vitamin B_{12} metabolism. Our evidence includes the identification of nonconservative mutations in *MMADHC* in each of seven patients with cblD defects and the demonstration that expression vectors containing the wild-type *MMADHC* gene rescue cblD function in fibroblast cell lines. The predicted MMADHC protein contains the putative cobalamin binding motif and a putative mitochondrial targeting sequence.

A complete analysis of the cblD defect requires an explanation of how mutations in the candidate gene lead to three distinct biochemical phenotypes. Although our data as described do not fully satisfy this requirement, our results suggest that the nature and location of the mutations are correlated with the biochemical phenotype, as illustrated in Figure 2B. Mutations found in the patients with cblD–methylmalonic aciduria are located toward the N-terminal part of the protein and consist of a nonsense mutation, a duplication, and a frame-shift deletion. Mutations found in the patients with cblD-homocystinuria are located toward the C-terminal part of the protein and consist of three missense mutations. Mutations found in the patients with the cblD-combined phenotype are located toward the C-terminal part of the protein and consist of a nonsense mutation, a splice-site deletion, and a frame-shift duplication.

Explaining these associations fully will require further studies. However, we offer the following speculations. The two patients with the cblD–methylmalonic aciduria defect carry mutations that are predicted to lead to truncated proteins of 19 amino acids in length (57_64del) or 53 amino acids in length (160C→T). We propose that the Met62 codon acts as a second start codon and leads to the reinitiation of translation,[18] resulting in the formation of a shorter functional cblD protein product that lacks the putative mitochondrial leader sequence but allows for normal methylcobalamin synthesis. This would explain the isolated methylmalonic aciduria without homocystinuria in Patients 3 and 4 and is in accordance with the rescue of methionine and methylcobalamin synthesis in cblD-homocystinuria and cblD-combined cell lines transfected with the mutant alleles 57_64del and 160C→T. In support of this "reinitiation of translation" hypothesis, a strong Kozak consensus sequence[19] occurs at cDNA positions 184–3 and 184+4 (A at position −3 and G at position +4) in the *MMADHC* gene.

Both patients with isolated homocystinuria carry missense mutations (545C→A, 746A→G, and 776T→C) that are sufficient to cause deficient synthesis of methylcobalamin. However, we speculate that these mutations allow for the formation of a modified protein with an intact functional domain for the synthesis of adenosylcobalamin.

The patients with combined homocystinuria and methylmalonic aciduria carry mutations (419dupA, 696+1_4del, and 748C→T) that are pre-

dicted to lead to premature stop codons. We presume that these mutations would result either in a defective protein lacking both functional domains or in a low, steady-state abundance of *MMADHC* mRNA by the mechanism of nonsense-mediated mRNA decay.

These interpretations imply that at least two functional domains are present in the cblD protein and that the nature and location of the mutations correlate with the biochemical phenotype. However, this hypothesis remains speculative until proved in more patients. An alternative hypothesis is that even under normal conditions, two proteins are produced. However, we find no evidence of two different transcripts, and a single transcript of *MMADHC* rescues both biochemical phenotypes. This gene can be added to the list of genes known to be associated with multiple phenotypes involving various subcellular compartments, such as the amnionless gene[20] and the multiple sulfatase gene.[21] To our knowledge, *MMADHC* is unique in that the three distinct biochemical phenotypes involve both the cytosolic and mitochondrial pathways.

The protein sequence of MMADHC is highly conserved among various mammalian species (Fig. 3). *MMADHC* is not a member of any previously identified gene family but was initially selected because of homology to a putative bacterial ABC transporter.

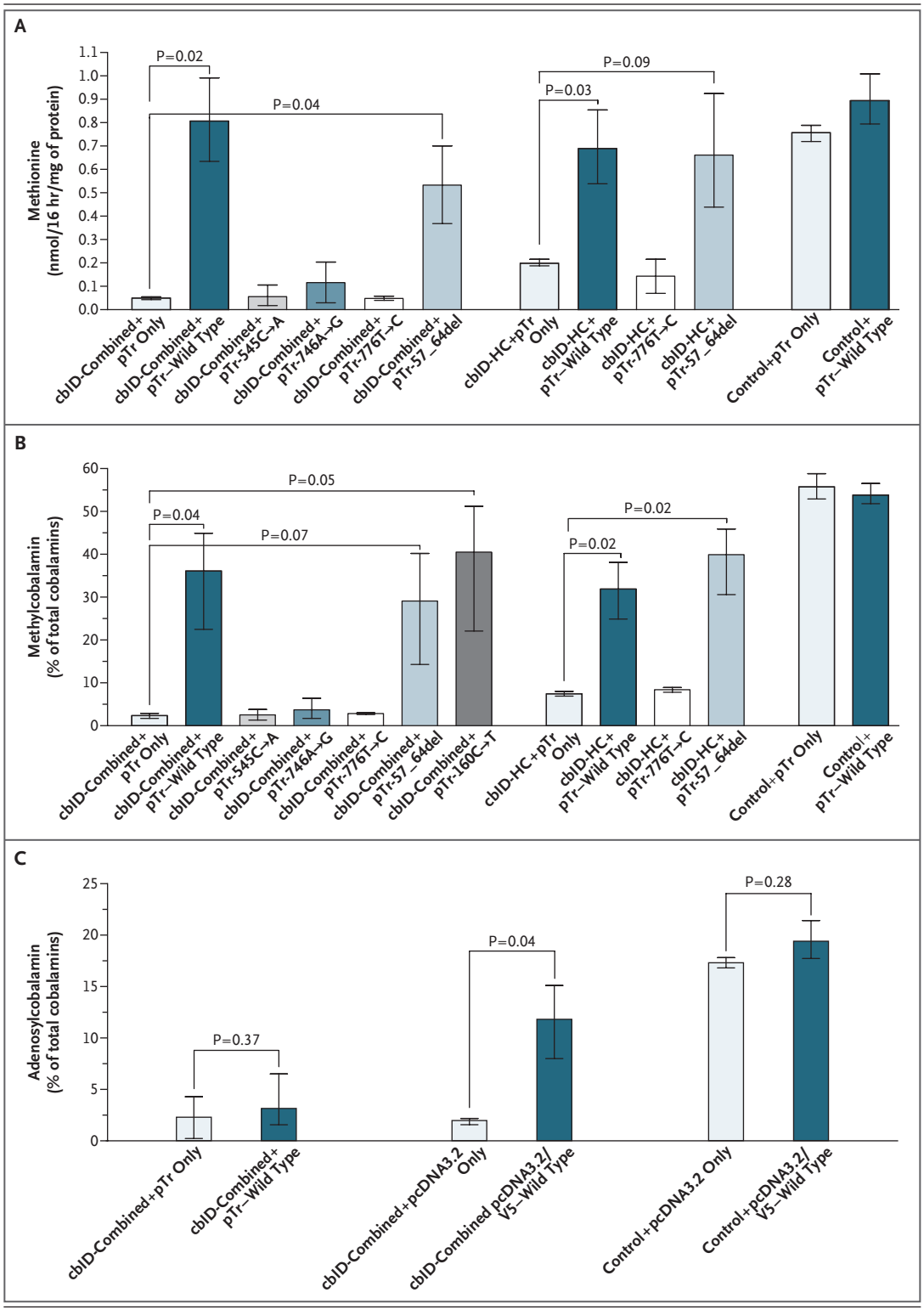
Although the segment of 91 amino acids between residues 78 and 168 shares 28% identity and 46% similarity with a putative ATPase component of an ABC transporter from *Salmonella enterica* (YP_218380), the cblD protein lacks critical motifs of ABC transporters such as Walker A, Walker B, and an ABC signature.[22] This makes it unlikely that the cblD protein is a classic ABC transporter. ABC transporters are a diverse family of proteins with multiple functions; MMADHC may be a new type of ABC transporter or may be involved in a complex that facilitates transport in a fashion similar to that of ABC transporters.

It is not known how cobalamin enters the mitochondria in humans. Both active diffusion and passive diffusion have been suggested.[23,24] In some bacteria, cobalamin transport is facilitated by two processes. First, it is transported through the outer membrane by the BtuB transporter,[15] which is mediated by TonB,[25] a protein that couples energy from the proton motive force to the transport of cobalamin. Second, it is transported through the inner membrane by the BtuFCD ABC transport system.[22] The C-terminal region of the human cblC protein (MMACHC) folds in a manner similar to that of TonB from *Escherichia coli*,[26] and the human cblD protein MMADHC, the existence of which we have predicted, shares some sequence identity with an ABC transporter from *S. enterica*. It is tempting to speculate that the *MMACHC* and *MMADHC* genes may have evolved to carry out functional roles that are similar to those of their bacterial counterparts, by forming structurally similar proteins.

In conclusion, we studied seven patients with the cblD defect in vitamin B_{12} metabolism. We found that mutations in a gene we designated *MMADHC* are responsible for this defect, and we demonstrated that various mutations are associated with each of the three biochemical phenotypes of the disorder.

Figure 4 (facing page). Expression of Wild-Type and Mutant Alleles of Human *MMADHC* (Methylmalonic Aciduria, cblD Type, and Homocystinuria) in Immortalized cblD-Combined, cblD-Homocystinuria, and Control Fibroblasts.

Cells from Patient 7 with the cblD-combined phenotype (homozygous for 696+1_4delGTGA), Patient 2 with the cblD-homocystinuria phenotype (cblD-HC) (compound heterozygous for missense mutations 545C→A and 746A→G), and one control were immortalized and used for transfection experiments. Transient transfection was performed by means of electroporation with pTracer (pTr) vectors containing an *MMADHC* allele: the wild-type allele, one of the three missense alleles (545C→A, 746A→G, or 776T→C) associated with cblD-HC, or the frame-shift deletion 57_64delCTCTTTAG or the nonsense mutation 160C→T associated with cblD–methylmalonic aciduria. Background activity was measured in cells transfected with empty pTracer vector (pTr only). Panel A shows the formation of methionine from [^{14}C]formate, and Panels B and C show the synthesis of methylcobalamin, and adenosylcobalamin, respectively, from [^{57}Co]cyanocobalamin. Transfection of the cblD-combined cell line and the control cell line was also performed with the pcDNA3.2/V5 vector containing wild-type *MMADHC*. Background activity was measured in cells transfected with empty pcDNA3 vector (pcDNA3.2 only). The data are means, and the I bars indicate the range of results of single determinations from each experiment. For Panels A and B, the values are from three replicate experiments, and P values are shown only for comparisons indicating rescue of function. For Panel C, values for the pTracer vector are from seven replicate experiments, and those for the pcDNA3.2 vector are from three replicate experiments.



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